The effect of benzo[a]pyrene on metabolic activation of anticancer drug ellipticine in mice

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OBJECTIVES: The aim of this study was to investigate a role of cytochrome P450 (CYP) and peroxidase in ellipticine oxidative activation in two mouse strains differing in expression of NADPH:CYP reductase (POR) [the HRN (Hepatic Cytochrome P450 Reductase Null) mice, in which POR is deleted in hepatocytes and its wild-type (WT)

counterpart], and in levels of CYP1A1/2 and cytochrome b_5 that were modulated by treatment of these mouse models with a CYP1A inducer, benzo[a]pyrene (BaP).

METHODS: Ellipticine-DNA adducts were detected by ^{32}P-postlabeling. HPLC was employed for the separation and characterization of ellipticine metabolites.

RESULTS: Hepatic microsomes of HRN and WT mice activate ellipticine to form ellipticine-derived DNA adducts. A 2.2- and 10.4-fold increase in amounts of ellipticine-derived DNA adducts formed by liver microsomes was caused by exposure of HRN and WT mice to BaP, respectively. The results found and utilization of NADPH and arachidonic acid, cofactors of CYP- and cyclooxygenase (COX)-dependent enzyme systems, respectively, as well as inhibitors of CYP1A1/2 and 3A, demonstrate that the CYP1A and 3A enzymes play a major role in ellipticine activation in liver microsomes. In addition, the COX enzyme is important in ellipticine activation in liver of HRN mice.

CONCLUSION: The CYP1A and 3A enzymes activate ellipticine mainly in liver of WT mice, whereas peroxidase COX plays this role in liver of HRN mice. Treatment of mice with BaP increases an impact of CYP1A on ellipticine activation. A pattern of expression levels of these enzymes plays a crucial role in their impact on this process.

KEYWORDS

ellipticine; anticancer drug; benzo[a]pyrene; cytochrome P450 1A1/2; induction; DNA adducts

Abbreviations:

AHR – aryl hydrocarbon receptor

48 α-NF – α-naphthoflavone

49 BaP – benzo[a]pyrene

50 BPDE – BaP-7,8-dihydrodiol-9,10-epoxide

51 COX– cyclooxygenase

52 CYP – cytochrome P450

53 dG-N^2-BPDE – 10-(deoxyguanosin-N^2-yl)-7,8,9-trihydroxy-7,8,9,10-tetrahydrobenzo-

54 [*a*]pyrene

55 DMSO – dimethyl sulfoxide

56 EROD – ethoxyresorufin *O*-deethylase

57 GAPDH - glyceraldehyde phosphate dehydrogenase

58 HPLC – high performance liquid chromatography

59 HRN – Hepatic Cytochrome P450 Reductase Nul

60 i.p. - intraperitoneally

61 mEH – microsomal epoxide hydrolase

62 NADPH – nicotinamidadeninedinucleotide phosphate (reduced)

63 POR – NADPH:cytochrome P450 reductase

64 RAL – relative adduct labeling

65 r. t. – retention time

66 S.D. – standard deviation

67 SDS-PAGE – sodium dodecyl sulfate-polyacrylamide gel electrophoresis

68 TLC – thin-layer chromatography

69 UV – ultraviolet

70 WT – wild-type

71

INTRODUCTION

Ellipticine (5,11-dimethyl-6*H*-pyrido[4,3-*b*]carbazole, Fig. 1) and its derivatives are efficient anticancer compounds that function through multiple mechanisms participating in cell cycle arrest and initiation of apoptosis [for a summary see (Auclair, 1987; Garbett & Graves, 2004; Stiborova et al. 2001, 2006c, 2011; Kizek et al. 2012)]. Ellipticine was found (i) to arrest cell cycle progression due to modulation of levels of cyclinB1 and Cdc2, and phosphorylation of Cdc2 in human mammary adenocarcinoma MCF-7 cells, (ii) to initiate apoptosis due to formation of toxic free radicals, stimulation of the Fas/Fas ligand system and modulation of proteins of Bcl-2 family in several tumor cell lines, (iii) to induce an increase in wild-type p53 and the rescue of mutant p53 activity, and (iv) to initiate the mitochondria-dependent apoptotic processes [for a summary see (Kuo et al. 2005a,b; 2006; Stiborova et al. 2011; Kizek et al. 2012)]. Ellipticine also activates the p53 pathway in glioblastoma cells; its impact on these cancer cells depends on the p53 status. In a U87MG glioblastoma cell line expressing p53wt, ellipticine provoked an early G0/G1 cell cycle arrest, whereas in a U373 cell line expressing p53mt it caused arrest in S and G2/M phase (Martinkova et al. 2010).

The predominant molecular mechanisms of ellipticine's biological effects were suggested to be (i) intercalation into DNA and (ii) inhibition of topoisomerase II (Auclair, 1987; Garbett & Graves, 2004; Stiborova et al. 2011). We also showed that this antitumor agent forms covalent DNA adducts after its enzymatic activation with cytochromes P450 (CYP) and peroxidases (Stiborova et al. 2001, 2004, 2006a, 2007a,b, 2008, 2010, 2011, 2012b,c,d; Kotrbova et al. 2011; Kizek et al. 2012), suggesting an additional DNA-damaging effect of ellipticine.

96 Of the CYP enzymes investigated, human CYP3A4, followed by CYP1A1 and
97 1A2 are the most active enzymes oxidizing ellipticine to 12-hydroxy- and 13-
98 hydroxyellipticine, the reactive metabolites that dissociate to ellipticine-12-ylium and
99 ellipticine-13-ylium which bind to DNA (Stiborova et al. 2004, 2007a, 2011, 2012b,d).
100 The CYP1A isoforms also efficiently form the other ellipticine metabolites, 7-hydroxy-
101 and 9-hydroxyellipticine, which are the detoxification products (Fig. 1). Recently we
102 have found that cytochrome b_5 alters the ratio of ellipticine metabolites formed by
103 CYP1A1, 1A2 and 3A4. While the amounts of the detoxification metabolites (7-
104 hydroxy- and 9-hydroxyellipticine) were either decreased or not changed with added
105 cytochrome b_5, 12-hydroxy-, 13-hydroxyellipticine and ellipticine N^2-oxide increased
106 considerably. The change in amounts of metabolites resulted in an increased
107 formation of covalent ellipticine-DNA adducts, one of the DNA-damaging
108 mechanisms of ellipticine antitumor action (Kotrbova et al. 2011; Stiborova et al.
109 2012b). In addition, we found that levels of the DNA adduct formed by 13-
110 hydroxyellipticine also increased if this ellipticine metabolite was conjugated with
111 sulfate or acetate by human sulfotransferases 1A1, 1A2, 1A3 and 2A1, or *N,O*-
112 acetyltransferases 1 and 2 (Moserova et al. 2008; Kotrbova et al. 2011; Stiborova et
113 al. 2012b) (Fig. 1). Of the mammalian peroxidases, human cyclooxygenase (COX)-2,
114 ovine COX-1, bovine lactoperoxidase and human myeloperoxidase efficiently
115 generated ellipticine-derived DNA adducts (Poljakova et al. 2006, Stiborova et al.
116 2007a).

117 The same ellipticine-derived DNA adducts that were found in *in vitro* incubations
118 of ellipticine with DNA and enzymes activating this drug, were generated also *in vivo*,
119 in several tissues of mice and rats exposed to ellipticine. In both animal models,

ellipticine-DNA adduct formation was mediated mainly by CYP1A and 3A enzymes, but a role of peroxidases in several organs was also proved (Stiborova et al. 2003a, 2003b, 2007b, 2008, 2010). The ellipticine-DNA adducts were also found in several cancer cell lines expressing CYP and/or peroxidase enzymes and in DNA of rat mammary adenocarcinoma *in vivo* (Borek-Dohalska et al. 2004; Poljakova et al. 2007, 2009, 2011, 2013; Martinkova et al. 2009; Stiborova et al. 2011).

All these results indicate that expression levels of CYP and peroxidase enzymes metabolizing ellipticine seem to be crucial for antitumor, cytostatic and genotoxic activities of this drug in individual tissues. However, the actual impacts of these enzymes *in vivo* depend on several additional factors (Stiborova et al. 2008, 2011). One of them might be the presence of various patterns of individual CYPs and peroxidases and/or even the presence of other proteins influencing their activities such as cytochrome b_5 in target and non-target tissues (Stiborova et al. 2008, 2011, 2012b; Kotrbova et al. 2011; Kizek et al. 2012).

Therefore, in this study we have used two mouse strains, in which four different patterns of CYP1A1, 1A2, POR and cytochrome b_5, the enzymes that are crucial to dictate ellipticine metabolism, were expressed. Namely, beside the HRN (Hepatic Cytochrome P450 Reductase Null) mouse strain, in which NADPH:CYP reductase (POR), the unique electron donor to CYPs is deleted specifically in hepatocytes, resulting in the loss of most hepatic CYP function (Henderson et al. 2003; Arlt et al. 2008; Stiborova et al. 2008) and its wild-type (WT) counterpart, we have also utilized these mouse strains in which the levels of CYP1A1/2 enzymes and cytochrome b_5 protein were modulated by their treatment with a strong CYP1A1/2 inducer, benzo[a]pyrene (BaP) (Arlt et al. 2008, 2012; Hodek et al. 2011; Phillips & Venitt,

144 2012). The use of such animal models can shed more light on the impact of these
145 biotransformation enzymes on metabolic activation and/or detoxification of ellipticine
146 in organisms.

147 The electrochemical method of Western blotting was used to estimate
148 cytochrome b_5, CYP1A1/2 and POR protein expression in hepatic microsomes of
149 used mouse models, whereas formation of ellipticine-derived DNA adducts or
150 ellipticine oxidative metabolites catalyzed by these microsomes were analyzed by the
151 ^{32}P-postlabeling or HPLC methods, respectively (Stiborova et al. 2001, 2006a,c,
152 2011).

153

154 **MATERIAL AND METHODS**

155 *Animal treatment*

156 Mouse model strains used in this study were identical to those used in our previous
157 works (Arlt et al. 2008, 2012). All experiments were approved by, and conducted in
158 accordance with, the National Institute of Health standards for the care and use of
159 experimental animals and the University of Cincinnati Medical Center Institutional
160 Animal Care and Use Committee. HRN (*Por*$^{lox/lox}$ + *Cre*^{ALB}) mice on a C57BL/6
161 background (CXR Bioscience Ltd., Dundee, UK) used in this study were derived as
162 described previously (Henderson et al. 2003). Mice homozygous for loxP sites at the
163 *Por* locus (*Por*$^{lox/lox}$) were used as wild-type (WT). BaP was dissolved in corn-oil at a
164 concentration of 12.5 mg/ml. Groups of female HRN and WT mice (3 months old, 25-
165 30 g) were treated intraperitoneally (*i.p.*) with 125 mg/kg body weight (n=3) of BaP
166 daily either for 1 day (group I) or 5 days. Control mice (n=3) received corn-oil only
167 either for 1 day or for 5 days. Animals were sacrificed 24 hours after the last dose

(Arlt et al. 2008, 2012). Mouse livers were removed, snap frozen and stored at –80ºC until analysis.

Preparation of hepatic microsomes

Hepatic microsomes from HRN and WT mice, untreated or treated with BaP as described above, were isolated as described previously (Stiborova et al. 2011, 2012a; 2012b). Pooled microsomal fractions were used for further analyses.

Preparation of CYP1A1 and POR antibodies

Recombinant rat CYP1A1 protein was purified to homogeneity from membranes of *Escherichia coli* transfected with a modified *CYP1A1* cDNA (Stiborova et al. 2002). Rabbit liver POR was purified as described (Arlt et al. 2003). Leghorn chickens were immunized subcutaneously three times (with one week interval) with rat recombinant CYP1A1 and rabbit hepatic POR antigens (0.1 mg/animal) emulsified in complete Freund's adjuvant for the first injection and in incomplete adjuvant for boosters. Immunoglobulin fraction was purified from pooled egg yolks using fractionation by polyethylene glycol 6000 (Stiborova et al. 2002; Hodek et al. 2013).

Determination of CYP1A1/2, POR and cytochrome b_5 protein levels in hepatic microsomes

Immunoquantitation of hepatic microsomal CYP1A1, CYP1A2 and POR was done essentially as described previously using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Stiborova et al., 2002, 2006b; Arlt et al. 2008). CYP and POR were probed with the chicken anti-rat CYP1A1 and chicken anti-rabbit POR polyclonal antibodies as reported (Stiborova et al. 2002, 2006b; Arlt et al. 2008). The antibody against rat recombinant CYP1A1 recognizes both CYP1A1 and CYP1A2 in mouse liver microsomes. Rat recombinant CYP1A1 and CYP1A2 (in Supersomes™,

Gentest Corp., USA) and rabbit POR were used as positive controls to identify protein bands in microsomal samples. Western blot analysis was also used to determine expression of cytochrome b_5; 75 µg microsomal protein was subjected to SDS-PAGE using 15% gel. After migration, proteins were transferred onto polyvinylidene difluoride membranes. Cytochrome b_5 protein was probed with rabbit polyclonal anti-cytochrome b_5 antibody (1:750; ab69801; Abcam, MA, USA) overnight at 4°C. Glyceraldehyde phosphate dehydrogenase (GAPDH; 1:750; Millipore, MA, USA) was used as loading control. The antigen-antibody complex was visualized with an alkaline phosphatase-conjugated goat anti-rabbit IgG antibody and 5-bromo-4-chloro-3-indolylphosphate/nitrobluetetrazolium as chromogenic substrate (Stiborova et al. 2002, 2006b; Arlt et al. 2008, 2012). The detection limit was 0.005 pmol CYP1A1 per lane (Stiborova et al. 2002, 2006b; Arlt et al. 2008) and 0.01 pmol for the other enzymes and cytochrome b_5.

Determination of CYP1A and POR enzymatic activity in hepatic microsomes

The hepatic microsomal samples were characterized for CYP1A activity using 7-ethoxyresorufin *O*-deetylation (EROD) (Stiborova et al. 2002, 2006b; Arlt et al. 2008). The activity of POR was measured as reported previously (Arlt et al. 2003).

Microsomal incubations

Incubation mixtures used to generate DNA adduct formation by ellipticine *in vitro* consisted of 50 mM potassium phosphate buffer (pH 7.4), 1 mM NADPH, pooled hepatic microsomal fraction (n=3) (0.5 mg protein) from either untreated (control) HRN or WT mice or those treated with BaP (see above), 0.1 mM ellipticine (dissolved in 7.5 µl methanol) and 0.5 mg of calf thymus DNA in a final volume of 750 µl. Incubations were also carried out in the presence of a COX cofactor, arachidonic acid

(Eling et al. 1992; Stiborova et al. 2005). Mixtures then contained 0.1 mM arachidonic acid as cofactor instead of NADPH, and additionally 5 mM magnesium chloride. Incubations were carried out at 37 ºC for 30 minutes; ellipticine-DNA adduct formation was found to be linear up to 30 min of incubation (Stiborova et al. 2001). Control incubations were carried out (*i*) without microsomes, (*ii*) without NADPH or arachidonic acid, (*iii*) without DNA and (*iv*) without ellipticine. After the incubation, DNA was isolated by a standard phenol-chloroform extraction method.

Incubation mixtures used to form the ellipticine metabolites contained 50 mM potassium phosphate buffer (pH 7.4), 1 mM $NADP^+$, 10 mM D-glucose 6-phosphate, 1 U/ml D-glucose 6-phosphate dehydrogenase (NADPH-generation system), 0.2 mg protein of pooled hepatic microsomal fraction and 10 μM ellipticine (dissolved in 5 μl methanol) in a final volume of 500 μl. The reaction was initiated by adding the substrate. In the control incubation, ellipticine was omitted from the incubation mixture. After incubation in open glass tubes (37 ºC, 20 min) the reaction was stopped by adding 100 μl of 2 M NaOH. The oxidation of ellipticine is linear up to 30 min of incubation (Stiborova et al. 2006a). After incubation, 5 μl of 1 mM phenacetine in methanol was added as an internal standard and the ellipticine metabolites were extracted twice with ethyl acetate (2 x 1 ml). Analyses of ellipticine metabolites were performed by HPLC as described (Stiborova et al. 2004, 2006a). Recoveries of ellipticine metabolites were around 95% in the presence of microsomes without a CYP cofactor (NADPH-generation system).

DNA adduct detection by ^{32}P-postlabeling analysis

DNA adducts formed by ellipticine were determined by ^{32}P-postlabeling analysis using the nuclease P1 enrichment version and DNA adducts were resolved by thin-layer chromatography (TLC) (Stiborova et al. 2003a, 2004, 2007a). After chromatography, TLC sheets were scanned using a Packard Instant Imager (Dowers Grove, USA) and DNA adduct levels (RAL, relative adduct labeling) were calculated as described (Stiborova et al. 2003a, 2004, 2007a). Results were expressed as DNA adducts/10^8 nucleotides.

Inhibition studies

The following chemicals were used to inhibit the activation of ellipticine to form DNA adducts in the presence of mouse hepatic microsomes: α-naphtoflavone (α-NF), which inhibits CYP1A1 and 1A2, ketoconazole, an inhibitor of CYP3A (Rendic & DiCarlo, 1997; Arlt et al. 2004; Stiborova et al. 2005), and α-lipoic acid, which inhibits POR (Slepneva et al. 1995). Inhibitors were dissolved in 7.5 μl of methanol, to yield final concentrations of 0.1 mM in the incubation mixtures. Mixtures were then incubated at 37 ºC for 10 min with NADPH prior to adding ellipticine, and then incubated for a further 30 min at 37 ºC. After the incubation, DNA was isolated as described above.

Statistical analyses

For statistical data analysis we used Student's *t*-test. All *P*-values are two-tailed and considered significant at the 0.05 level.

RESULTS AND DISCUSSION

Expression of hepatic CYP1A1, 1A2, POR and cytochrome b_5 in HRN and WT mice is induced by BaP

Hepatic microsomes isolated from both control (untreated) mice and mice treated with BaP were used in experiments evaluating their efficacies to activate ellipticine to species forming ellipticine-derived DNA adducts and/or to detoxify this compound to metabolites that are excreted. First, expression of CYP1A1/2, POR and cytochrome b_5, the enzymes that all dictate metabolic activation and/or detoxification of ellipticine was analyzed.

As shown in our previous study (Arlt et al. 2008), CYP1A1 and 1A2 enzymes are constitutively expressed in livers of untreated HRN and WT mice, with HRN mice having marginally higher levels (1.4-fold) than WT mice (Fig. 2). BaP was capable to effectively induce expression of both CYPs in livers of HRN and WT mice. Whereas exposure of these mice to BaP resulted in 8.9-fold higher levels of CYP1A1/2 in livers of WT mice, its inducing effect was higher in HRN mice; a 17.8-fold increase in CYP1A1/2 protein expression was caused by BaP in this mouse strain. The increase in CYP1A1/2 levels in the WT mice was associated with a strong increase in CYP1A1/2 marker activity, ethoxyresorufin *O*-demethylation (EROD) (Fig. 2C). Whereas CYP1A1/2 activity (EROD) was not detectable in untreated HRN mice, it was found in HRN mice treated with BaP. Its levels were, however, 3.4-fold lower than in WT mice treated with BaP. These findings are in concordance with previous studies, showing that expression of CYP1A1/2 is up-regulated by the aryl hydrocarbon receptor (AHR) and BaP can bind to and activate AHR, thereby enhancing metabolic activation of several genotoxic carcinogens, including BaP itself (Arlt et al. 2008; Hodek et al. 2011; Phillips & Venitt, 2012).

The expression of POR was detected in liver of WT mice, while as expected, its expression in liver of HRN mice was very low, but still detectable by the Western

286 blotting method (Fig. 2B). POR activity measured with cytochrome c as a substrate
287 was, however, not detectable in hepatic microsomes of HRN mice (Fig. 2D).
288 Surprisingly, the expression level of POR was also slightly induced in liver of both WT
289 and HRN mice treated with BaP (Fig. 2B); a 1.2- and 1.4-fold increase in POR protein
290 expression was mediated by treating the WT and HRN mice with BaP, respectively.
291 Likewise, POR enzyme activity was detectable in hepatic microsomes from HRN
292 mice treated with BaP (Fig. 2D).

293 Interestingly, beside induction of expression of these enzymes by BaP, the
294 levels of cytochrome b_5 protein has also been found to be induced by treating both
295 mouse strains with BaP; a 1.6- and 1.7-fold increase in cytochrome b_5 protein levels
296 was produced by BaP in livers of HRN and WT mice, respectively (Fig. 3) (Arlt et al.
297 2012).

298 *Exposure of HRN and WT mice to BaP increases activation of ellipticine to form*
299 *ellipticine-derived DNA adducts*

300 The activation of ellipticine by hepatic microsomes of HRN and WT mice was
301 analyzed by the ^{32}P-postlabeling technique (Fig. 4). In the presence of NADPH, a
302 cofactor of POR- and CYP-dependent enzyme systems, the *ex vivo* incubations of
303 hepatic microsomes of untreated (control) HRN and WT mice and mice treated with
304 BaP with ellipticine and DNA led to activation of this drug to form ellipticine-derived
305 DNA adducts (Fig. 4). The DNA adduct pattern generated by ellipticine consisted of
306 at least two adducts (spots 1 and 2 in Figure 4), which were identical to those formed
307 *in vivo* in mice and rats treated with ellipticine (Stiborova et al. 2003a, 2008), each
308 formed by 13-hydroxyellipticine (Fig. 4C) or 12-hydroxyellipticine (Fig. 4D)
309 metabolites that are generated by CYP and peroxidase (see Fig. 1). An additional

ellipticine-derived DNA adduct, spot A, was found in DNA that had been incubated with ellipticine and mouse hepatic microsomes (Fig. 4A), predominantly in microsomes isolated from HRN mice (Table 1). This ellipticine-DNA adduct was not formed by either human or rat hepatic microsomes (Stiborova et al. 2001, 2003b, 2004) or in mice (Fig. 4E and F) or rats *in vivo* (Stiborova et al. 2003a, 2007b, 2008). In incubations containing hepatic microsomes of HRN and WT mice treated with BaP, an additional adduct spot, corresponding to the 10-(deoxyguanosin-N^2-yl)-7,8,9-trihydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (dG-N^2-BPDE) adduct that is the major product of reaction of BaP metabolite BaP-7,8-dihydrodiol-9,10-epoxide (BPDE) with DNA *in vitro* and *in vivo* (Arlt et al. 2008; Phillips & Venitt, 2012) was also detected (Fig. 4B). This finding indicates that residual BaP is present in microsomes isolated from livers of HRN and WT mice, and is activated by CYP1A1 in combination with microsomal epoxide hydrolase (mEH) to form this adduct. Control incubations without microsomes were free of the dG-N^2-BPDE adduct, ellipticine-DNA adducts 1 and A, but ellipticine-derived adduct 2 was always detected (data not shown). This finding is consistent with our previous results showing that this adduct is formed also non-enzymatically (Stiborova et al. 2001, 2003b, 2004, 2007a). In the presence of microsomes without NADPH, beside ellipticine-DNA adducts 2, a low but detectable amount of the adduct 1 (Table 1) and the dG-N^2-BPDE adduct was also found (data not shown).

Surprisingly, levels of ellipticine-derived DNA adducts formed in *ex vivo* incubations of ellipticine and DNA with hepatic microsomes from HRN mice in the presence of NADPH were only 1.4-fold lower than amounts formed by hepatic microsomes from WT mice (Fig. 5 and Table 1), even though POR expression in

334 livers of HRN mice was two orders of magnitude lower. This finding indicates that
335 ellipticine activation should, at least partially, be catalyzed also by enzymes, whose
336 activities are not dependent on POR (Stiborova et al. 2008). Beside peroxidases that
337 were found to activate ellipticine (see Stiborova et al. 2007a and the results shown
338 below), the CYP2S1 enzyme, which is abundantly expressed in several tissues
339 (Downie et al. 2005; Saarikoski et al. 2005; Bui et al. 2009a) might be such an
340 enzyme. Namely, it was shown that it catalyzes the oxidation of compounds having
341 polycyclic aromatic structures similar to ellipticine without participation of POR (Bui et
342 al. 2009a,b). Whereas a role of a COX peroxidase is investigated in this study, the
343 participation of CYP2S1 in ellipticine activation still awaits further examination.
344 Therefore, the efficiency of CYP2S1 to oxidize ellipticine is planned to be investigated
345 in our future work.

346 Treatment of both HRN and WT mice with BaP resulted in increased levels of
347 ellipticine-derived DNA adducts formed in *ex vivo* incubations of ellipticine with
348 hepatic microsomes of these mice relative to microsomes of untreated mice. A 2.2-
349 and 10.4-fold increase in formation of amounts of ellipticine-derived DNA adducts
350 was caused by exposure of HRN and WT mice to BaP, respectively, of them the
351 levels of adduct 1 were predominantly increased. In contrast, a low (adduct 2 in both
352 HRN and WT mice) or no increase (adduct A in HRN mice) in DNA adduct formation
353 was produced by BaP (Table 1).

354 Since CYP1A, 3A and peroxidase enzymes were found to activate ellipticine
355 (Stiborova et al. 2001, 2003b, 2004, 2007a), we investigated the modulation of
356 ellipticine-derived DNA adduct formation by cofactors and selective inhibitors of these

357 enzymes using hepatic microsomes isolated from both untreated (control) mouse
358 strains and the animals treated with BaP.

359 α-Lipoic acid, a selective inhibitor of POR (Slepneva et al. 1995), decreased
360 ellipticine-DNA adduct formation by 25-75% with mouse hepatic microsomes.
361 Therefore, some activity in both HRN and WT mice is dependent on POR. α-NF, an
362 inhibitor of CYP1A1 and 1A2 (Rendic & DiCarlo, 1997), decreased the levels of
363 ellipticine-DNA adducts generated by hepatic microsomes of untreated HRN and WT
364 mice as well as of WT mice treated with BaP, to essentially equal extent (to 40% in
365 microsomes of WT and BaP-treated WT mice and to 50% in those of HRN mice), but
366 had no inhibition effect of activation of ellipticine with microsomes of HRN mice
367 treated with BaP (Fig. 5, Table 1). This finding proved a role of CYP1A in activation of
368 WT and HRN mice, and mainly in WT mice in which CYP1A expression was induced
369 with BaP. Induction of CYP1A in HRN mice resulted, however, in opposite process;
370 induced CYP1A enzymes seem to increase ellipticine detoxification (see also below).
371 Ketoconazole, a selective inhibitor of CYP3A enzymes (Rendic & DiCarlo, 1997;
372 Ueng et al. 1997), inhibited formation of ellipticine-DNA adducts in hepatic
373 microsomes of untreated (control) HRN and WT mice, by ~60%, but its effect was
374 much lower in hepatic microsomes of BaP-treated HRN and WT mice, only by ~10%
375 (Fig. 5, Table 1). These results point additionally to CYP3A enzymes as having a role
376 in ellipticine-DNA adduct formation in mouse livers, but their contributions to this
377 process was decreased by induction of CYP1A1 with BaP.

378 Arachidonic acid, a cofactor for COX-dependent oxidation (Eling et al. 1990,
379 1992; Stiborova et al. 2004, 2005; Arlt et al. 2006), mediated formation of DNA

adducts 1 and 2 by ellipticine in hepatic microsomes of all mice used, but was much less effective than NADPH (Fig. 5, Table 1).

Ellipticine metabolites formed in hepatic microsomes from all mouse strains used in this work were analogous; 9-hydroxy-, 12-hydroxy-, 13-hydroxy, 7-hydroxyellipticine and N^2-oxide of ellipticine were formed (Fig. 6). However, the amounts of individual metabolites in HRN and WT mice, either control (untreated) or treated with BaP, were different. In the case of untreated mice, 9-hydroxyellipticine levels were only one sixth, while the amounts of 13-hydroxy- and 12-hydroxyellipticine, were about one half in incubations with HRN microsomes compared with the levels in incubations with WT microsomes. Exposure of both HRN and WT mouse strains to BaP resulted in an increase in formation of 9-hydroxy- and 7-hydroxyellipticine, the metabolites that are considered to be detoxification products. More than 3- and 23-fold higher amounts of 9-hydroxy-and 7-hydroxyellipticine were formed by induction of CYP1A with BaP in WT mice, respectively. Likewise, treatment of HRN mice with BaP resulted in 3.8- and 5.5-fold higher levels of 9-hydroxy-and 7-hydroxyellipticine generated by hepatic microsomes of these mice, respectively (Fig. 6). This result is consistent with findings of the former studies, where CYP1A1 and 1A2 were found to be the major enzymes forming these metabolites (Stiborova et al. 2004; Kotrbova et al. 2006, 2011). However, treatment of WT mice with BaP also resulted in up to 2.5-fold increased levels of 13-hydroxy- and 12-hydroxyellipticine (Fig. 6). Such an increase might result not only from induction of CYP1A enzymes by BaP, but also from induction of cytochrome b_5 by this compound (see Fig. 3). Indeed, cytochrome b_5 alters the ratio of ellipticine metabolites formed by CYP1A1 and 1A2 increasing the amounts of 13-hydroxy- and

12-hydroxyellipticine (Kotrbova et al. 2011). The increased formation of both these ellipticine metabolites was associated with increased levels of ellipticine-DNA adducts that are generated by reaction of DNA with these two metabolites (see Figs. 5, 6 and Table 1 and the results shown in Kotrbova et al. 2011 and Stiborova et al. 2012b). In the case of HRN mice, no increase in 13-hydroxy- and 12-hydroxyellipticine formation was found by treatment of these mice with BaP; a pattern of CYP1A1/2, POR and cytochrome b_5 seems not to be appropriate to favor formation of these metabolites. Nevertheless, ellipticine-derived DNA adducts were increased when HRN mice were treated with BaP (Figs. 5, 6 and Table 1). There might be at least one reason for the above finding; activities of additional enzymes, beside CYPs, which are present in hepatic microsomes of HRN mice and are capable of activating ellipticine such as COX (Stiborova et al. 2007a) are induced by BaP. Indeed, an increase in formation of ellipticine-derived DNA adducts, predominantly the adduct 1, in *ex vivo* incubations of ellipticine with microsomes of HRN mice treated with BaP and arachidonic acid, a cofactor of COX, was found. Formation of adduct 1 from ellipticine13-ylium generated from COX-dependent formation of 6,13-didehydroellipticine (ellipticine mehylene-imine) (Fig. 1) should therefore also occur in hepatic microsomes of HRN mice treated with BaP.

CONCLUSION

Using mouse models in which expression of enzymes metabolizing ellipticine were modulated either by deletion in liver (POR) or by induction with BaP (CYP1A1/2 and cytochrome b_5), we confirm a major role of CYP1A and 3A and participation of COX in ellipticine activation in these mice. Treatment of mice with BaP increases an

impact of CYP1A on ellipticine activation. The results found also emphasize that a pattern of expression levels of these enzymes plays a crucial role in their impact on this process. Therefore, this study forms the basis to further predict the susceptibility of human cancers to ellipticine and suggests this alkaloid for treatment in combination with CYP gene transfer (Ma & Waxman, 2007; Lu et al. 2009) increasing the anticancer potential of this pro-drug.

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Table 1. DNA adduct formation by ellipticine activated by hepatic microsomes from either untreated (control) Hepatic Cytochrome P450 Reductase Null (HRN) and wild-type (WT) mice (**A**) or mice treated with benzo[a]pyrene (BaP) (**B**)

A	RAL[a] (mean/10^8 nucleotides)			
	Spot 1	Spot 2	Spot A	Total
HRN mice hepatic microsomes + NADPH	5.9±0.3	2.3±0.2	6.7±0.3	14.9±0.8
HRN mice hepatic microsomes + NADPH + α-lipoic acid	1.2±0.1	1.7±0.1	0.9±0.1	3.8±0.3
HRN mice hepatic microsomes + NADPH + α-NF	2.8±0.2	2.2±0.2	1.0±0.1	6.0±0.5
HRN mice hepatic microsomes + NADPH + ketoconazole	2.5±0.2	2.0±0.2	1.0±0.1	5.5±0.4
HRN mice hepatic microsomes + arachidonic acid	2.9±0.2	2.4±0.2	ND	5.3±0.4
HRN mice hepatic microsomes without cofactor	0.2±0.04	1.9±0.2	ND	2.1±0.2
WT mice hepatic microsomes + NADPH	13.0±0.9	3.2±0.2	4.8±0.4	21.0±1.5
WT mice hepatic microsomes + NADPH + α-lipoic acid	7.6±0.6	2.7±0.2	0.7±0.1	11.0±0.9
WT mice hepatic microsomes + NADPH + α-NF	6.5±0.5	2.7±0.2	1.2±0.1	10.4±0.8
WT mice hepatic microsomes + NADPH + ketoconazole	6.0±0.5	2.1±0.2	1.0±0.1	8.1±0.7
WT mice hepatic microsomes + arachidonic acid	5.7±0.4	4.6±0.3	ND	10.3±0.8
WT mice hepatic microsomes without cofactor	0.3±0.02	2.0±0.2	ND	2.3±0.2

B	RAL[a] (mean/10^8 nucleotides)			
	Spot 1	Spot 2	Spot A	Total
BaP-HRN mice hepatic microsomes + NADPH	22.6±1.3	5.7±0.3	4.2±0.2	32.5±1.6
BaP-HRN mice hepatic microsomes + NADPH + α-lipoic acid	21.5±1.0	3.4±0.2	ND	24.9±1.3
BaP-HRN mice hepatic microsomes + NADPH + α-NF	30.6±1.5	6.7±0.3	ND	37.3±1.9
BaP-HRN mice hepatic microsomes + NADPH + ketoconazole	23.1±1.1	5.6±0.3	ND	28.7±1.4
BaP-HRN mice hepatic microsomes + arachidonic acid	10.1±0.5	2.3±0.1	ND	12.4±0.7
BaP-HRN mice hepatic microsomes without cofactor	0.2±0.01	1.9±0.1	ND	2.1±0.1
BaP-WT mice hepatic microsomes + NADPH	181.7±6.3	8.3±0.4	28.7±1.2	218.7±8.9
BaP-WT mice hepatic microsomes + NADPH + α-lipoic acid	82.9±4.0	5.3±0.3	4.1±0.2	92.3±4.4
BaP-WT mice hepatic microsomes + NADPH + α-NF	83.5±4.1	4.1±0.2	3.8±0.2	91.4±4.4
BaP-WT mice hepatic microsomes + NADPH + ketoconazole	164.3±8.1	7.4±0.4	26.5±1.2	198.2±9.1
BaP-WT mice hepatic microsomes + arachidonic acid	8.1±0.4	2.6±0.1	ND	10.7±0.5
BaP-WT mice hepatic microsomes without cofactor	0.5±0.03	2.5±0.1	ND	3.0±0.1

[a] Mean RAL (relative adduct labeling) ± standard deviation (S.D.) of four determinations (duplicate analyses of two independent *in vitro* incubations). ND - not detected (the detection limit of RAL was $1/10^{10}$ nucleotides). For adduct spots 1,2 and A, see Figure 4. NADPH - nicotinamidadeninedinucleotide phosphate (reduced); α-NF - α-naphthoflavone.

Figure 1. Scheme of ellipticine metabolism by cytochrome P450 (CYP) enzymes showing the identified metabolites and those proposed to form DNA adducts. The compounds shown in brackets were not detected under the experimental conditions and/or not yet structurally characterized. The CYP enzymes predominantly oxidizing ellipticine shown in the figure were identified in our previous studies (Stiborova et al. 2004, 2012b; Kotrbova et al. 2011). Reactions (rea) 1, 2 and 3 are reactions leading to ellipticine-13-ylium from 13-hydroxyellipticine, 13-hydroxyellipticine sulfate and 13-hydroxyellipticine acetate, respectively.

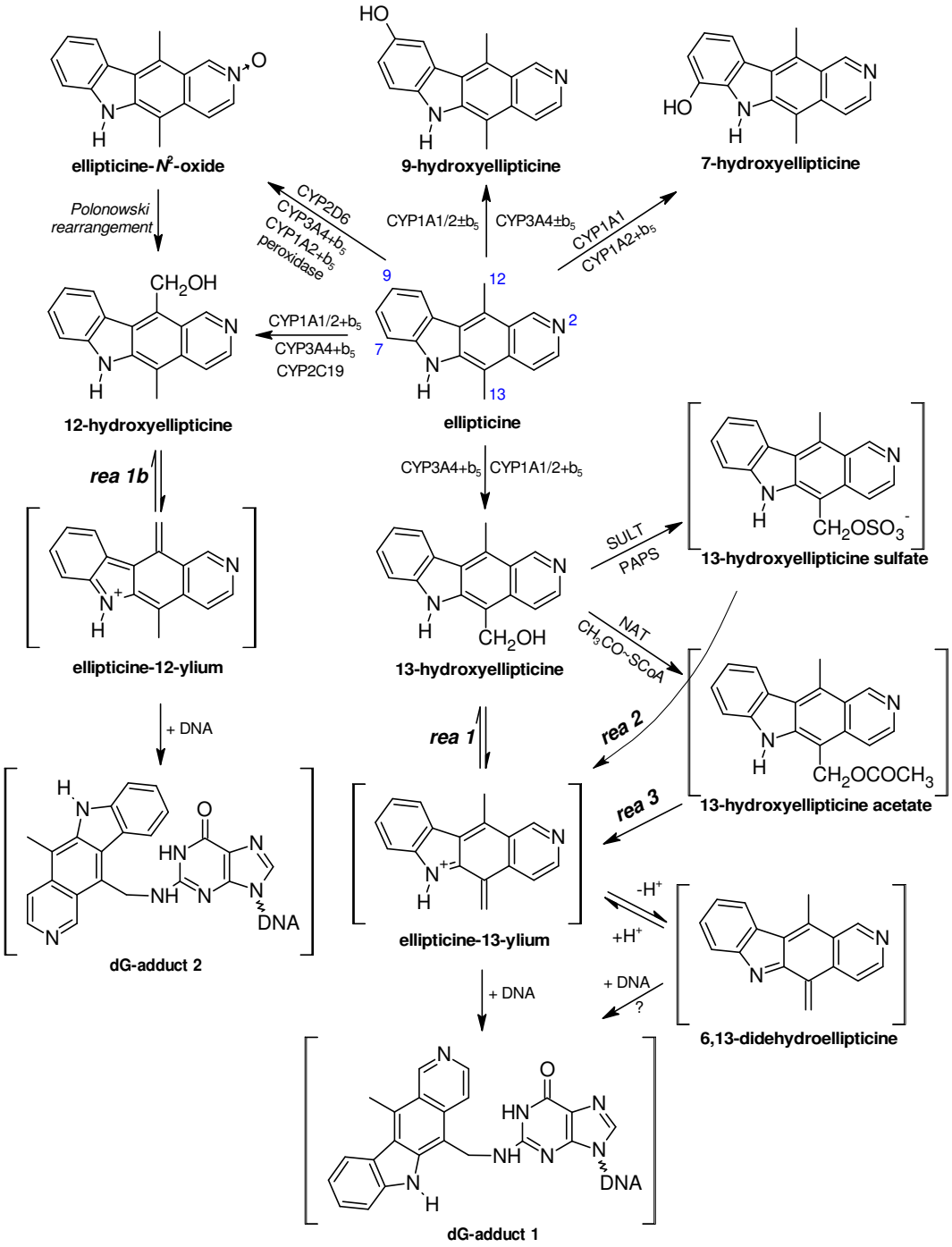

Figure 2. Expression of cytochrome P450 (CYP) 1A1/2 (**A**) and NADPH:cytochrome P450 reductase (POR) (**B**) and their enzymatic activity [7-ethoxyresorufin *O*-deetylation (EROD) (**C**) and POR activity (**D**)] in livers of Hepatic Cytochrome P450 Reductase Null (HRN) and wild-type (WT) mice, control (untreated), or treated intraperitoneally (*i.p.*) with 125 mg benzo[a]pyrene (BaP)/kg body weight daily for five days. Inset in A and B: immunoblots of microsomal CYP1A1/2 and POR from each mouse group, stained with antibody against rat CYP1A1 and rabbit POR, respectively. Pooled hepatic microsomal samples were used for analyses as described in Material and Methods. Values are given as means ± standard deviations (S.D.) (n=3). Values significantly different from untreated mice: *P<0.05, ***P<0.001. ND= not detectable. AU - arbitrary units. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as loading control (adopted from Arlt et al. 2008).

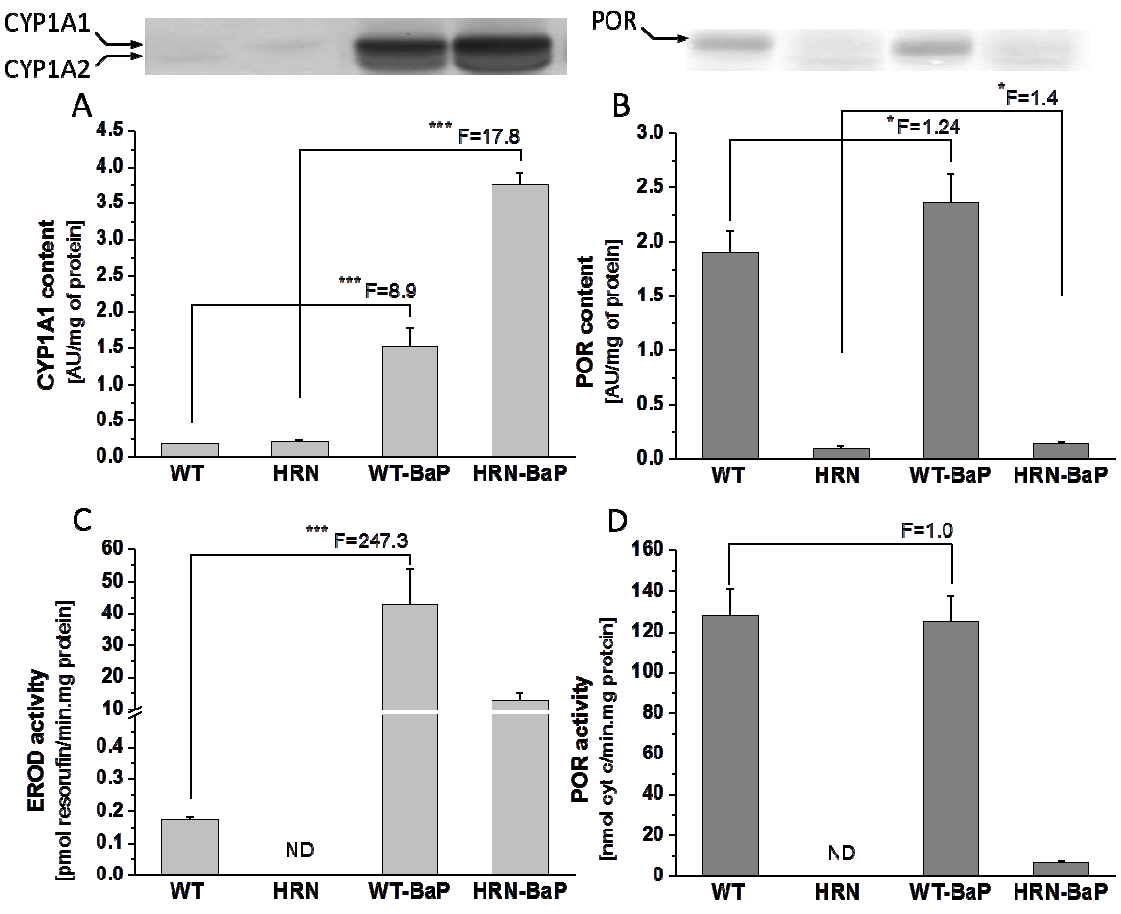

Figure 3. Expression of cytochrome b_5 (cyt b_5) in livers of Hepatic Cytochrome P450 Reductase Null (HRN) and wild-type (WT) mice, control (untreated) mice or mice treated intraperitoneally (*i.p.*) with 125 mg/kg body weight (bw) benzo[a]pyrene (BaP) for 24 hours. Pooled hepatic microsomal samples (n=3) were used for analyses as described in Material and Methods. Values significantly different from untreated mice: ***P<0.001. AU - arbitrary units. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as loading control (adopted from Arlt et al. 2012).

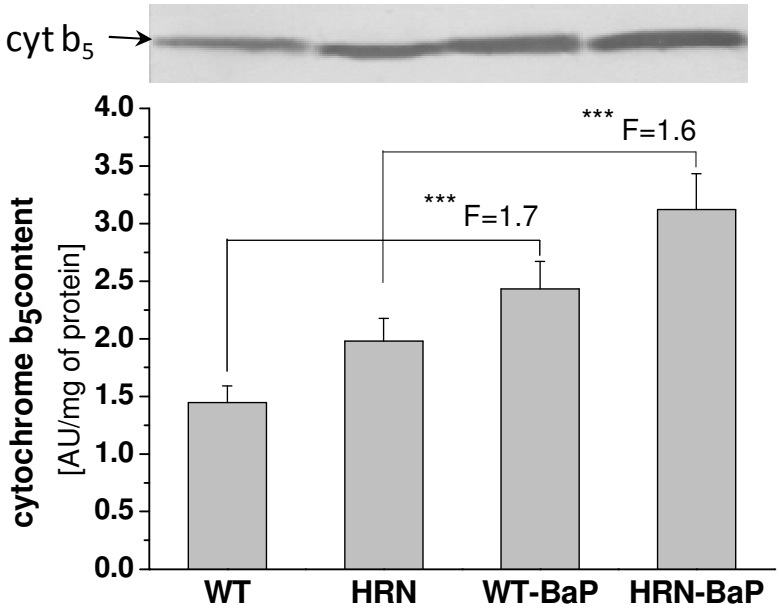

Figure 4. Autoradiographs of thin layer chromatography (TLC) maps of ^{32}P-labeled digests of calf thymus DNA reacted with ellipticine and hepatic microsomes from wild-type (WT) mice (**A**), with those of Hepatic Cytochrome P450 Reductase Null (HRN) mice treated with benzo[a]pyrene (BaP) (**B**), from calf thymus DNA reacted with 13-hydroxyellipticine (**C**) (Stiborova et al. 2004) and 12-hydroxyellipticine (**D**) (Stiborova et al. 2007a) and of DNA from livers of WT (**E**) and HRN (**F**) mice treated with 10 mg ellipticine/kg body weight (Stiborova et al. 2008). Analyses were performed by the nuclease P1 version of the ^{32}P-postlabeling assay.

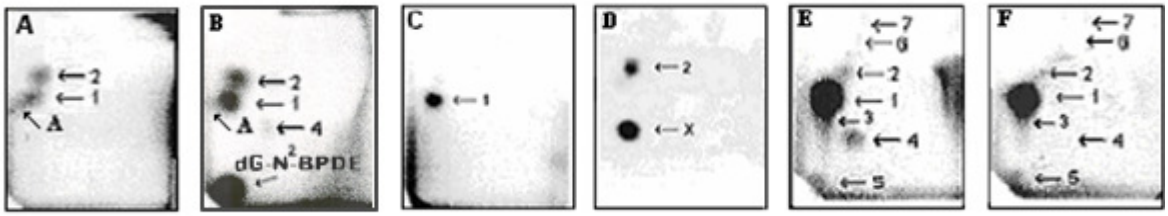

Figure 5. DNA adduct formation by ellipticine activated with microsomes isolated from livers of untreated Hepatic Cytochrome P450 Reductase Null (HRN) and wild-type (WT) mice **(A)** and from those treated with BaP **(B)** as determined by thin layer chromatography (TLC) ^{32}P-postlabeling. F = fold higher DNA adducts levels in microsomes from WT mice compared to HRN mice. Columns: Mean RAL (relative adduct labeling) ± standard deviations (S.D.) shown in the figure represent total levels of DNA adducts of four determinations (duplicate analyses of two independent *in vitro* incubations). Values significantly different from HRN mice:*P<0.05, **P<0.01, ***P<0.001. Control = without cofactor; AA = arachidonic acid; α-NF = α-naphthoflavone; α-LA = α-lipoic acid. ND = not detected.

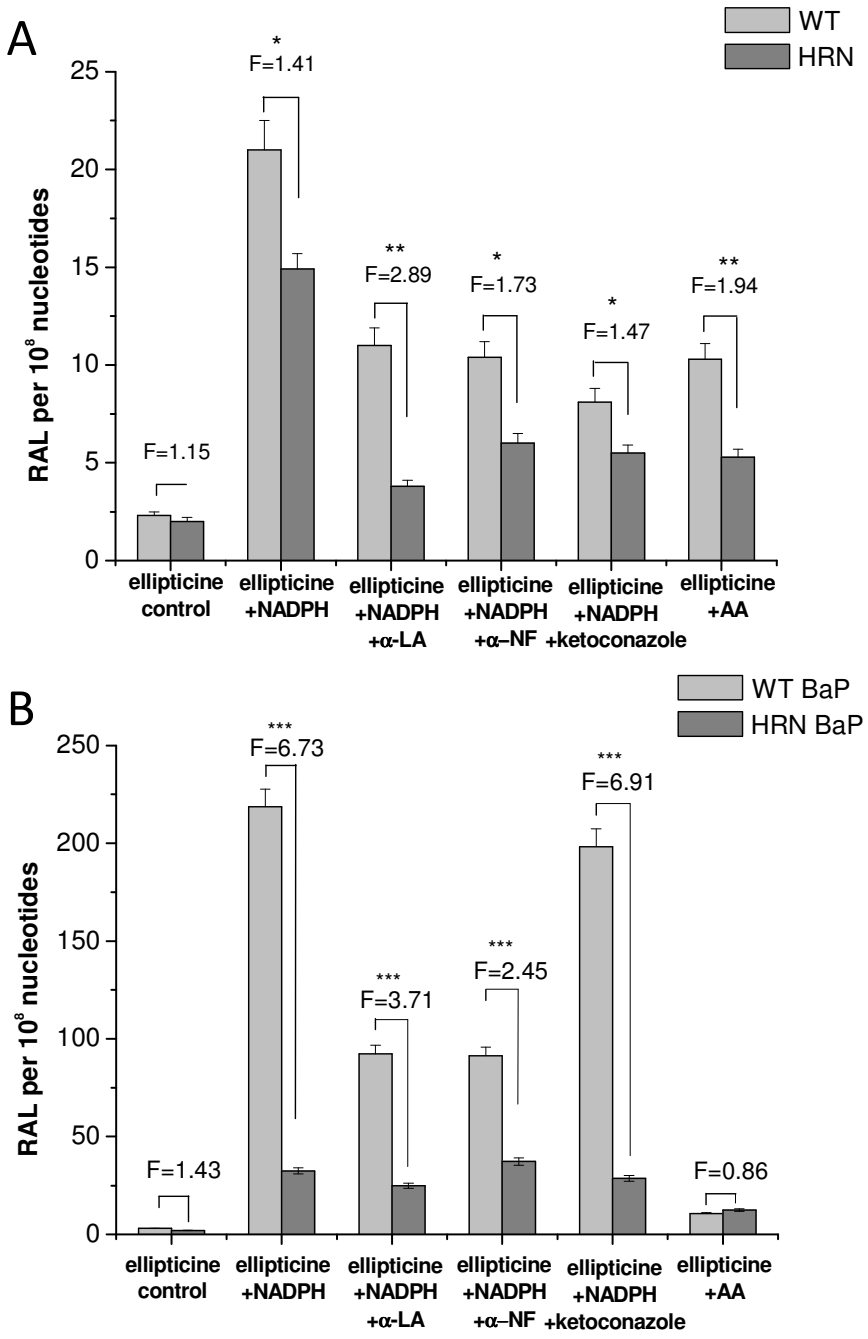

Figure 6. Levels of ellipticine metabolites formed by hepatic microsomes (0.2 mg protein) of Hepatic Cytochrome P450 Reductase Null (HRN) and wild-type (WT) mice from 10 μM ellipticine and by hepatic microsomes of HRN and WT mice treated intraperitoneally (*i.p.*) with 5 × 125 mg of benzo[a]pyrene (BaP) per kg of body weight. Levels of ellipticine metabolites were determined by high performance liquid chromatography (HPLC) (Stiborova et al. 2004, 2006a) and are averages ± standard deviations of triplicate incubations. Values significantly different from untreated mice: *P<0.05, **P<0.01, ***P<0.001.